Hydroa Vacciniforme Is Associated with Increased Numbers of Epstein–Barr Virus–Infected γδT Cells

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Hydroa vacciniforme (HV) is a rare photosensitivity disorder of childhood associated with Epstein–Barr virus $(EBV)^+$ T-cell infiltration. We have summarized clinical manifestations of HV, and analyzed EBV^+ T-cell subsets as well as EBV DNA load in lymphocyte fractions, in comparison with hypersensitivity to mosquito bites (HMB), an EBV-associated T/natural killer (NK) lymphoproliferative disorder. We found that 31 of 33 (93.9%) HV lesions were composed of EBV^+ T cells and reactive EBV^- cytotoxic T cells, without significant $CD56^+$ cell infiltration, whereas many $CD56^+$ cells were present in 8 of 9 (88.9%) HMB lesions. Of 13 (20.6%) HMB patients with or without HV, 12 (92.3%) showed increased percentages (>32%) of NK cells in the peripheral blood, whereas in the 16 patients with HV alone, 14 (87.5%) showed no increase. Of the 11 HV patients, 10 (90.9%) had increased percentages (>5%) of circulating γδT cells, with a mean value of 15.7 ± 2.9%, and the γδT-cell fractions contained larger amounts of EBV DNA than non-γδT-cell fractions. A triple-labeling method revealed that all three HV patients examined had increased percentages of $EBER^+$, T-cell receptor $(TCR)\gamma\delta^+$, and $TCR\alpha\beta^-$ cells. Our observations indicate that HV is associated with increased numbers of EBV^+ γδT cells, whereas HMB is associated with EBV^+ NK cells.

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INTRODUCTION

Epstein–Barr virus (EBV), a γ-herpesvirus, has a preferential tropism for B cells and epithelial cells. However, it has been recognized that EBV likely infects and causes the proliferation of not only B cells, but also T or natural killer (NK) cells, and induces EBV-associated T/NK lymphoproliferative disorders such as chronic active EBV infections and hemophagocytic syndrome (Rezk and Weiss, 2007; Ohshima *et al.*, 2008). Previous studies have shown that cutaneous disorders such as hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB) are closely associated with EBV-infected T or NK cells (Iwatsuki *et al.*, 1997, 1999, 2006; Verneuil *et al.*, 2010).

HV is a photosensitivity disorder of childhood characterized by herpetiform vesiculopapules on sun-exposed areas such as face, ears, and dorsal surfaces of hands. The cutaneous HV lesions contained various number of EBV-encoded small nuclear RNA $(EBER)^+$ T cells, together with larger numbers of $EBER^-$ cytotoxic T lymphocytes (CTLs) expressing T-cell intracellular antigen (TIA)-1 and granzyme B, whereas NK cells were absent or at background levels in HV lesions (Morizane *et al.*, 2005; Iwatsuki *et al.*, 2006).

Although no hematological abnormalities are usually found in HV patients, EBV DNA load was increased in the peripheral blood mononuclear cells (PBMCs; Iwatsuki *et al.*, 2006). In contrast, patients with severe HV usually present with ulcerative cutaneous lesions with dense inflammatory cell infiltrates reaching the subcutaneous tissue, and also have markedly increased levels of EBV DNA load in the peripheral blood. These patients often have complications such as fever, leucopenia, liver damage, and lymphadenitis, and may progress to fatal hemophagocytic syndrome and HV-like T-cell lymphoma (Kimura *et al.*, 2001, 2005; Iwatsuki *et al.*, 2006).

HMB is an EBV-associated T/NK lymphoproliferative disorder characterized by intense local skin reactions and systemic symptoms including high fever, lymphadenopathy, hepatosplenomegaly, and hemophagocytic syndrome (Tokura *et al.*, 2001; Asada *et al.*, 2005). These clinical symptoms are induced by mosquito bites, insect bites, or vaccination. Patients with HMB are usually associated with NK cell lymphocytosis with latent EBV infection (Tokura *et al.*, 2001; Iwatsuki *et al.*, 2006). HMB is often associated with HV-like eruptions. It is intriguing to note that typical HV

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Abbreviations: CTL, cytotoxic T lymphocyte; EBER, EBV-encoded small nuclear RNA; EBV, Epstein–Barr virus; HMB, hypersensitivity to mosquito bites; HV, hydroa vacciniforme; NK, natural killer; PBMC, peripheral blood mononuclear cell; TCR, T-cell receptor

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occurs worldwide, but the vast majority of patients with severe HV and HMB have been reported in Asian countries and Latin America (Magana *et al.*, 1998; Kimura *et al.*, 2001, 2005; Tokura *et al.*, 2001; Cho *et al.*, 2004; Asada *et al.*, 2005; Morizane *et al.*, 2005; Nitta *et al.*, 2005; Iwatsuki *et al.*, 2006; Rodriguez-Pinilla *et al.*, 2010).

Although previous observations suggest that the occurrence of HMB is closely related to EBV$^+$ NK cells (Tokura *et al.*, 2001), we still do not know which cell type has a pivotal role in the development of HV. Recently, we and other investigators have noticed that some patients with HV may have increased numbers of EBV$^+$ γδT cells in the peripheral blood (Kimura *et al.*, 2009; Tanaka *et al.*, 2011). In order to identify the T-cell subsets responsible for the development of HV, we have analyzed phenotypes of infiltrating cells in HV lesions, as well as the circulating T-cell subsets harboring latent EBV infection, in comparison with those in HMB.

RESULTS

Clinical manifestations of typical and severe HV

Of the 33 typical HV patients, 7 had complications with oculomucosal involvement such as conjunctival hyperemia and aphthous stomatitis, but neither internal nor hematological symptoms were observed in this group (Figure 1 and Table 1). In severe HV patients (Figure 1), however, 7 of 15 patients presented with systemic symptoms, including fever, lymph node swelling, liver damage, hemophagocytic syndrome, and other organ involvement such as esophageal erosions, myocardial infiltration, and colon ulcer. Of the 15 HMB patients, 9 presented with HMB alone in the observation period, and 6 had a history of HV or coexistent HV lesions. Many of the patients with HMB, with or without HV, experienced systemic symptoms, and one patient progressed to nasal-type NK lymphoma. In our series of patients with severe HV and HMB, at least three patients died of disease progression or intractable hemophagocytic syndrome, and two patients recovered from HMB with a decrease in EBV DNA load in the PBMCs.

EBV DNA load in typical and severe HV

EBV DNA levels in PBMCs were elevated in all 29 typical HV patients, with a mean value of 18,500 ± 5,100 copies per μg DNA, ranging from 110 to 79,500 copies per μg DNA (Figure 2). Severe HV patients had higher levels of EBV DNA in the PBMCs than typical HV: 30,200 ± 6,400 copies per μg DNA, ranging from 450 to 101,000 copies per μg DNA, the level of which was comparable to that in patients with HMB with or without HV. There was no significant difference in plasma EBV DNA levels between typical and severe HV: 2,400 ± 820 copies per ml in typical HV versus 6,500 ± 3,900 copies per ml in severe HV.

Cellular events in HV and HMB cutaneous lesions

Immunophenotypes of infiltrating cells in typical and severe HV lesions were distinct from those in HMB lesions. In HV lesions, ~5 to 20% of the infiltrates were positive for EBER. An NK cell marker, CD56, was absent or at a background level in 31 of 33 (93.9%) cutaneous lesions. In the remaining two cases with CD56$^+$ cell infiltration, it was difficult to identify the infiltrating cell type because a part of the circulating sCD3$^+$ T cells also expressed CD56 at various degrees. In addition to the EBER$^+$ cells, all HV lesions contained dense infiltration of EBER$^-$ cells expressing any T-cell markers, including CD3ε, CD45RO, CD4, and CD8, as well as the majority of infiltrating cells carrying cytotoxic molecules such as TIA-1 and granzyme B. Many CD3$^+$, T-cell receptor (TCR)β$^-$ T cells were present in two HV lesions examined (Supplementary Figure S1 online). In HMB lesions, however, in addition to a small number of EBER$^+$ cells and many EBER$^-$ CTLs, 8 of 9 HMB lesions contained considerable numbers of CD56$^+$ cells in the infiltrates. One patient with coexistent HV and HMB lesions showed a clear difference in CD56$^+$ cell infiltration between lesions: CD56$^+$ cell infiltration was absent in the HV lesions, but present in the HMB lesions (Figure 3).

Lymphocyte subsets in HV and HMB

No significant increase was observed in total lymphocyte numbers in the peripheral blood of HV or HMB patients, although the numbers of large granular lymphocytes (Figure 1) were increased in some cases. The percentages of sCD3$^-$ and CD56$^+$ NK cells in PBMCs were increased (to >32%) in 12 of 13 (92.3%) HMB patients with or without HV, whereas no such increase was observed in 14 of 16 (87.5%) patients with HV alone (Figure 4a and b). The mean values of sCD3$^-$ and CD56$^+$ NK cells were 48.6 ± 5.0% in the HMB group with or without HV, and 11.0 ± 2.5% in the HV group. In 10 of 11 (90.9%) HV patients examined, however, the percentages of γδT-cell receptor (TCRγδ)$^+$ cells were increased (to >5%), ranging from 7 to 36% of peripheral blood lymphocytes (Figure 4c). The mean percentage and absolute number of TCRγδ$^+$ cells in all HV patients was 15.7 ± 2.9% (vs. 2.1 ± 0.5% in normal individuals) and 266 ± 108 μl, respectively.

Clonality analysis of EBV$^+$ cells

Of the 11 samples tested by Southern blot analysis using EBV terminal repeat, EBV clonality was found in 8 samples, biclonal in 2 samples, and polyclonal in 1 sample (Table 2). In a patient with HMB associated with sCD3$^-$, CD8^{low+}, and CD56$^+$ NK lymphocytosis, at least two different T-cell clones showing a phenotype of EBV$^+$, sCD3$^+$, CD4$^+$, and CD56$^{+/-}$ were found in the short-term culture with 50 units ml^{-1} of recombinant IL-2 (Supplementary Figure S2 online). The two major EBV$^+$ clones expressed Vβ3 and Vβ13.2, respectively. These data, together with the results of lymphocyte subsets, indicate that HMB patients, but not HV patients, were associated with EBV$^+$ NK cell lymphocytosis with or without monoclonal proliferation, although the HMB patients may also possess EBV$^+$ T-cell clones.

EBV$^+$ γδT-cell subset in HV

As PBMCs from HV patients showed elevated percentages of TCRγδ$^+$ cells, we have focused on the EBV$^+$ γδT-cell subset in the development of HV. In fractionated lymphocyte

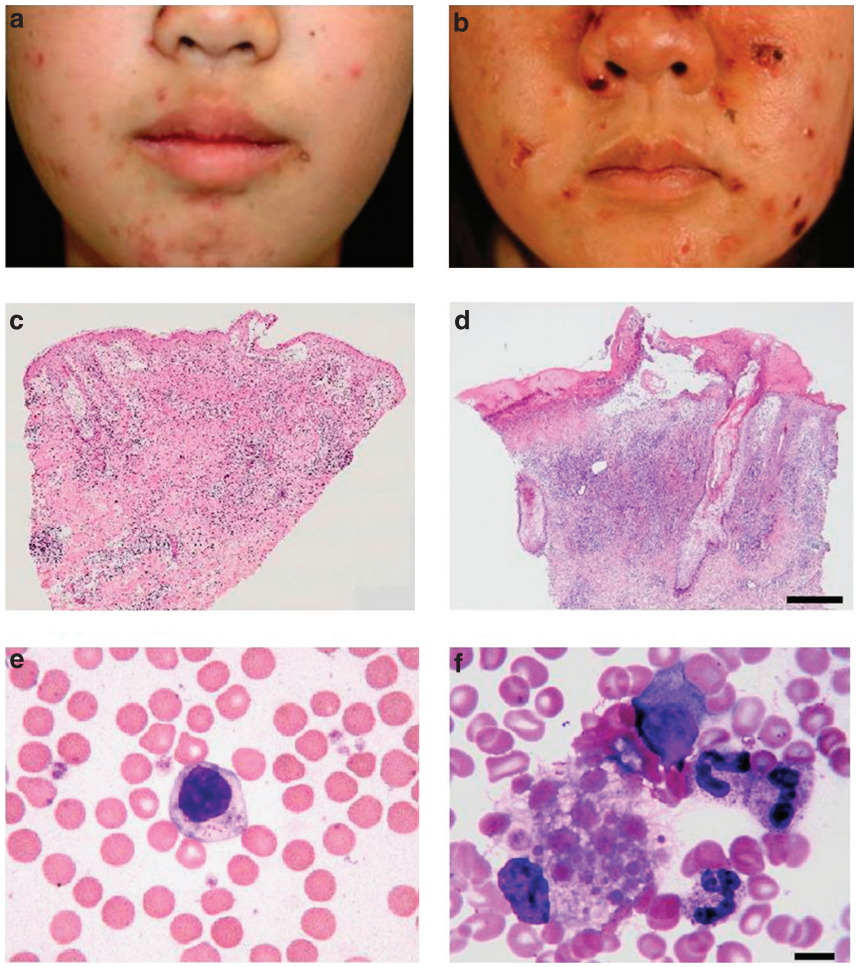

Figure 1. Clinicopathological features of typical and severe HV. Cutaneous lesions of (**a**) typical hydroa vacciniforme (HV) and (**b**) severe HV, showing (**c**) moderate lymphocytic infiltration in the upper dermis (hematoxylin/eosin stain), and (**d**) dense lymphocytic infiltration in the dermis and subcutaneous tissue (bar = 500 μm), respectively. (**e**, **f**) A large granular lymphocyte in peripheral blood cells, and a feature of hemophagocytosis in bone marrow in a severe HV patient (May–Giemsa stain; bar = 10 μm).

subsets of cases 1, 3, and 4, the TCRγδ$^+$ cell fractions contained higher EBV DNA load than the sCD3$^+$ cell fractions, including TCRγδ$^+$ as well as TCRαβ$^+$ and other cell types. In cases 5, 6, and 7, the TCRγδ$^+$ cell fractions contained significantly higher EBV DNA load than the TCRγδ$^-$ cell fractions (Table 2, $^*P<0.05$).

In three representative HV cases examined, the triple-labeling method detected EBER$^+$, TCRγδ$^+$, and TCRαβ$^-$ cells in high percentages, ranging from 57 to 68% of EBER$^+$ cells, whereas EBER$^+$, TCRγδ$^-$, and TCRαβ$^+$ cells were present in low percentages, ranging from 3 to 5% (Figure 4d). No increase in EBER$^+$ cells was found in sCD3$^-$ and CD19$^+$ B cells or in sCD3$^-$ and CD56$^+$ NK cell fractions. In contrast, 6 HMB patients (cases 8, 9, 11, 12, 13, and 14 in Table 2), with or without HV, had higher levels of EBV DNA load in the CD56$^+$ cell fractions than in the sCD3$^+$ T-cell fractions. Therefore, taken together with the results of the surface phenotypes (Figure 4), most HV and HMB patients harbored EBV$^+$ γδT cells, and EBV$^+$ NK cells, respectively, in the peripheral blood.

DISCUSSION

Immunohistological examination revealed that the histogenesis of HV was distinct from that of HMB: HV lesions were composed mainly of various percentages of EBER$^+$ cells, and EBER$^-$ cytotoxic T cells without CD56 expression, whereas HMB lesions contained considerable numbers of CD56$^+$ cells. As shown in Figure 3, a patient with coexistent HV and HMB showed distinct cellular infiltrates: CD56$^+$ cells are absent in the HV lesions, but present in the HMB lesions. HV lesions, therefore, are induced by EBER$^+$ T cells and a larger number of EBER$^-$ CTLs, without apparent involvement of NK cell infiltration. In contrast, 6 of 7 HMB patients (Figure 4b) revealed increased percentages (>32%) of sCD3$^-$ and CD56$^+$ NK cells in their peripheral blood, whereas the percentages of sCD3$^-$ and CD56$^+$ NK cells were slightly increased in only 2 of 16 HV patients. Furthermore, six HMB patients examined (Table 2) had higher levels of EBV DNA load in the CD56$^+$ cell fraction than in sCD3$^+$ T-cell fractions. These data, taken together with previous reports (Morizane *et al.*, 2005; Iwatsuki *et al.*,

Table 1. Clinical background of patients with HV and/or HMB

					CD56+ cells in skin lesion		Complications					
	Cases (M:F)	Age (y)	EBER+ cells in skin lesion		In HV lesion	In HMB lesion	Fever	LN swelling	Liver damage	HPS	Other organ involvements	
tHV	33 (16:17)	2–62 (mean 9.6)	1+	17	0/20	0/0	0	0	0	0	0	
			2+	8								
			3+	3								
			4+	0								
sHV	15 (7:8)	4–77 (mean 18.5)	1+	5	2/10	0/0	5	3	3	1	Esophageal erosions	1
			2+	5							Myocardial involvement	1
			3+	2							Colon erosions	1
			4+	3								
HMB	9 (6:3)	5–22 (mean 12.2)	1+	4	0/0	6/6	8	6	5	0	0	
			2+	2								
			3+	0								
			4+	1								
HV+HMB	6 (2:4)	3–34 (mean 12.3)	1+	1	0/3	2/3	5	2	4	1	Nasal-type lymphoma	1
			2+	2								
			3+	2								
			4+	0								
Total	63 (31:32)	2–77 (mean 12.4)			2/33	8/9	18	11	12	2		

Abbreviations: EBER, Epstein–Barr virus (EBV)-encoded small nuclear RNA; F, female; HMB, hypersensitivity to mosquito bites; HPS, hemophagocytic syndrome; HV, hydroa vacciniforme; LN, lymph node; M, male; sHV, severe hydroa vacciniforme; tHV, typical hydroa vacciniforme.
1+ Indicates 1% to <5% positivity in the infiltrates; 2+ indicates 5% to <25% positivity; 3+ indicates 25% to <50% positivity; and 4+ indicates ⩾50% positivity.

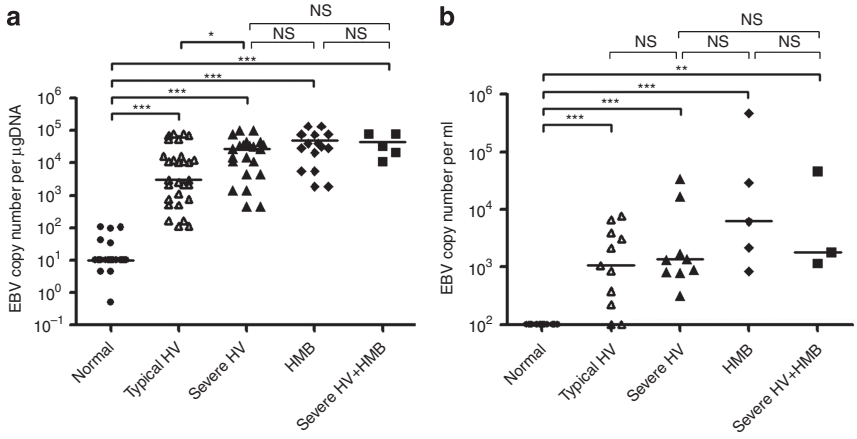

Figure 2. Epstein–Barr virus (EBV) DNA load in patients with hydroa vacciniforme (HV) and/or hypersensitivity to mosquito bites (HMB) in the peripheral blood mononuclear cells (PBMCs) and plasma. Patients with HV and/or HMB harbor significantly high levels of EBV DNA load in the (**a**) PBMCs and (**b**) plasma, as compared with normal individuals (**$P<0.01$, ***$P<0.001$). Among the patient groups, patients with severe HV, HMB, and HV + HMB show significantly higher levels of EBV DNA load in the PBMCs than patients with typical HV (*$P<0.05$; NS, not significant).

2006), indicate that cutaneous lesions of both typical and severe HV are induced by $EBER^+$ T cells, associated with a larger number of $EBER^-$ CTLs, without apparent involvement of NK cell infiltration, whereas the occurrence of HMB lesions is closely associated with increased numbers of $EBER^+$ NK cells (Tokura *et al.*, 2001).

Some patients with HMB, however, presented with HV simultaneously or at different periods in their clinical courses (Nitta *et al.*, 2005; Iwatsuki *et al.*, 2006).

This overlapping might be because of the presence of different EBV^+ T- and NK-cell clones responsible for the development of HV and HMB, respectively. Our hypothesis

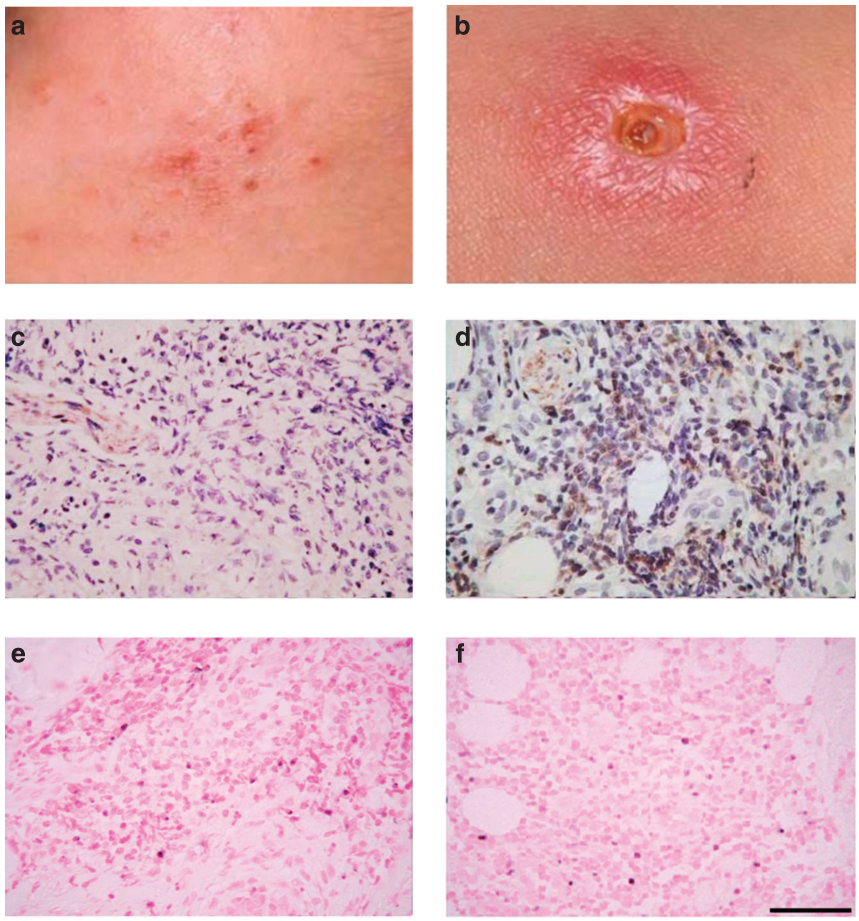

Figure 3. Distinct cellular infiltrates in hydroa vacciniforme (HV) and hypersensitivity to mosquito bites (HMB). This patient (case 12 in Table 2) presented with both (**a**) typical HV and (**b**) HMB lesions at the same time. (**c**) The HV lesion contains many T cells without CD56 expression (CD56 immunostain), (**d**) but the HMB lesion is composed of many CD56$^+$ cells. (**e, f**) A few EBER$^+$ cells are observed in both lesions (*in situ* hybridization; bar = 50 μm). (**c, d**) Note that both lesions contain peripheral nerves positive for CD56 (internal positive control).

may partly be supported by the fact that monoclonal proliferation of EBV$^+$ cells was found in 8 of 11 blood samples, but other 3 samples revealed biclonal or polyclonal proliferation. Furthermore, in one HMB patient with a dominant NK-cell clone, we detected at least two different EBV$^+$ T-cell clones in the short-term culture. This phenomenon is similar to the previous observations that established human T-cell leukemia virus type 1 (HTLV-1)$^+$ T-cell clones were not always derived from the dominant clone in the peripheral blood of the patients (Maeda *et al.*, 1985). Our data suggest that the EBV$^+$ cell fractions of the patients may constitute a mixture of one or a few dominant T/NK cell clones and some minor clones, the condition of which might account for the occurrence of various clinical symptoms (Kimura *et al.*, 2001, 2005; Iwatsuki *et al.*, 2006).

Although the development of HV lesions is closely associated with EBV$^+$ T-cell infiltration and reactive CTLs, HV patients usually reveal no increase in absolute numbers of sCD3$^+$ T cells. In the T-cell subsets, however, 10 of 11 (90.9%) HV patients had increased percentages of TCRγδ$^+$ cells in the PBMCs (Figure 4c), and higher levels of EBV DNA in the TCRγδ$^+$ cell fractions than TCRγδ$^-$ or total sCD3$^+$ cell fractions (Table 2). Furthermore, EBER$^+$, TCRγδ$^+$, and TCRαβ$^-$ cells were found in high percentages in three patients with HV by our triple-labeling flow cytometry. Therefore, our observations indicate that HV is pathogenically associated with increased numbers of circulating EBV$^+$ γδT cells. Because of technical limitation, there is no direct evidence for the involvement of EBV$^+$ γδT cells in HV lesions. So far, many CD3$^+$, TCRβ$^-$ T cells without CD56 expression, namely non-αβT cells, were present in two HV lesions examined (Supplementary Figure S1 online). Further research is also required to determine whether the sequential pathomechanism in the development of HV is initial sun exposure or photoprovocation, with the subsequent migration or activation of EBV$^+$ γδT cells, followed by CTL responses.

MATERIALS AND METHODS

Patients

Patients with "typical" HV fulfilled the following criteria: (1) repetitive vesiculopapular eruptions on exposed areas including

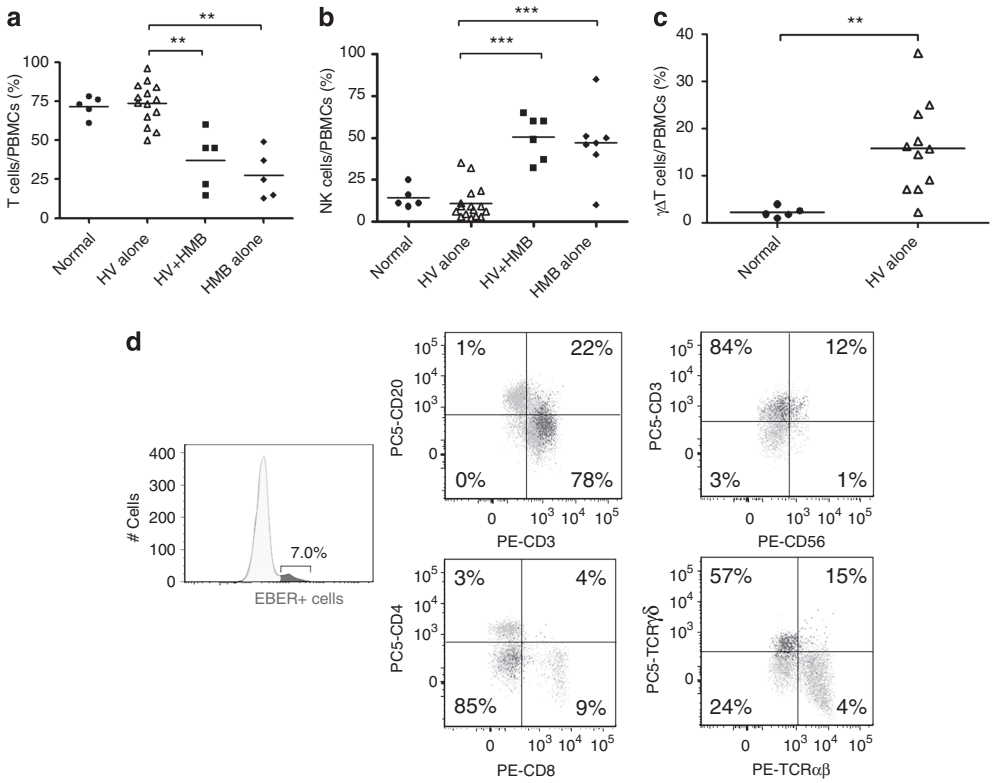

Figure 4. Lymphocyte subsets in peripheral blood mononuclear cells (PBMCs) from patients with hydroa vacciniforme (HV) and/or hypersensitivity to mosquito bites (HMB), and flow cytometric analysis on surface immunophenotypes and nuclear Epstein–Barr Virus-encoded small nuclear RNA (EBER). (**a–c**) Lymphocyte subsets in PBMCs from patients with HV and/or HMB. (**a, b**) The natural killer (NK) cell ($sCD3^-$ and $CD56^+$) percentages are significantly higher in a patient group of HMB than in a group of HV alone, indicating that HMB is closely associated with NK cell lymphocytosis. (**c**) The percentages of T-cell receptor (TCR)$\gamma\delta^+$ cells are increased in HV, (**a**) without significant increase in $sCD3^+$ T-cell percentages (**$P<0.01$, ***$P<0.001$). (**d**) Flow cytometric analysis on surface immunophenotypes and EBER. The majority of $EBER^+$ cells (red) in HV express a surface phenotype of $CD3^+$, $TCR\alpha\beta^-$, $TCR\gamma\delta^+$, $CD4^-$, $CD8^-$, and $CD56^-$ (case 1 in Table 2). The color reproduction of this figure is available in the *Journal of Investigative Dermatology* online.

the face, lips, cheeks, and extensor surfaces of the hands and arms, (2) histological features of reticulated degeneration of epidermis or blister formation associated with dense lymphocytic infiltration, and (3) the exclusion of hereditary photosensitivity disorders. In contrast, patients with ''severe'' HV presented with one or more of the following clinical and histopathological findings in addition to the HV-like eruptions: (1) high-grade fever, (2) liver damage, (3) ulcerative indurated lesions, and (4) edematous swelling of the cheeks, eyelids, ears, and lips. According to previous reports (Tokura *et al.*, 2001; Asada *et al.*, 2005), HMB was defined as ulceronecrotic skin lesions with infiltration of $EBER^+$ cells induced by mosquito bites, insect bites, or vaccination.

A series of 63 patients with HV and/or HMB were enrolled in this study. The median age of the patients was 8 years, with a range from 2 to 77 years, and the male to female ratio was 0.97. Skin biopsy materials and blood samples were obtained for diagnosis, and used in this study with the approval of ethics committee of Okayama University. Patient consent for experiments was not required because French laws consider human tissue left over from surgery as discarded material. All biopsy specimens were examined by routine hematoxylin/eosin-stained sections and immunostained sections with antibodies to CD3ε, CD4, CD8, CD20, CD45, CD56, and cytotoxic molecules such as TIA-1 and granzyme B. EBER was detected by *in situ* hybridization. According to the clinical and histopathological findings, 63 patients were classified into four groups: 33 patients with typical HV, 15 with severe HV, 9 with HMB alone, and 6 with both HMB and HV.

For double immunostaining, two biopsy specimens from HV were incubated with rabbit anti-human CD3 (Zymed Laboratories, South San Francisco, CA) and monoclonal mouse anti-human TCRβ (clone: G-11, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. The specimens were then incubated with biotinylated goat antimouse immunoglobulin (Dako, Glostrup, Denmark), followed by FITC-conjugated F(ab') fragment of goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories). The stained sections were observed using a fluorescence microscope (Olympus BX50, Tokyo, Japan).

Assay for EBV DNA load

After analyzing 14 blood samples from the patients, we reclassified them into three groups: HV alone ($n=7$), HMB alone ($n=3$), and HV + HMB ($n=4$), because of the overlapping clinical symptoms on the blood sampling. For EBV DNA load analysis, DNA was extracted from 1×10^6 PBMCs using a QIAamp Blood Kit (Qiagen, Hilden, Germany), and real-time quantitative PCR was performed with a fluorogenic probe. The EBV DNA load was calculated as copy number per μg of PBMC DNA.

Table 2. EBV DNA load in fractionated lymphocyte subsets

Case/ sex/age	Age at onset	Diagnosis	EBV clonality	Lymphocyte subsets in PBMCs		EBV DNA, copies per μg DNA			
				sCD3+ cell (%)	sCD56+ cell (%)	CD3+	CD19+	CD56+	TCRγδ+
1/M/4	3	sHV	Monoclonal	73	3	46,730	9,090	6,400	190,100
2/F/18	10	sHV	ND	96	6	55,431	37,536	84,110	ND
3/M/8	5	tHV	Monoclonal	58	17	16,380	ND	860	101,210
4/F/5	?	tHV	Biclonal	76	11	172,182	147,492	48,652	327,753
5/M/6	5	tHV	ND	74	6		TCRγδ$^-$ PBC 486		54,400
6/F/23	3	tHV	Monoclonal	84	5		TCRγδ$^-$ PBC 101		2,900
7/M/3	1	tHV	ND	85	3		TCRγδ$^-$ PBC 870		31,600
8/M/5	4	HMB	Monoclonal	15	10	11,288	18,423	86,361	ND
9/M/8	3	HMB	Polyclonal	25	50	7,428	17,083	89,352	ND
10/M/9	6	HMB	Monoclonal	37	51		TCRγδ$^-$ PBC 90,000		5,800
11/M/34	20	HV+HMB	Monoclonal	60	37	4,250	16,200	30,577	ND
12/F/3	2	HV+HMB	Biclonal	45	32	330	3,300	23,400	ND
13/F/6	2	HV+HMB	Monoclonal	15	60	3,288	1,866	35,252	ND
14/M/4	3	HV+HMB	Monoclonal	22	65	21,352	3,200	171,741	ND

Abbreviations: EBV, Epstein–Barr virus; F, female; HMB, hypersensitivity to mosquito bites; HV, hydroa vacciniforme; M, male; ND, not done; PBC, peripheral blood cell; PBMC, peripheral blood mononuclear cell; sHV, severe HV-like eruptions associated with high-grade fever, liver damage, indurated nodules, high titer against EBV, high EBV DNA copy number, and high mortality compared with typical HV; TCR, T-cell receptor; tHV, typical HV; ?, no data.

Lymphocyte subset analysis

To determine the cell types harboring EBV genomes, PBMCs were fractionated into CD3$^+$, CD19$^+$, CD56$^+$, TCRαβ$^+$, and TCRγδ$^+$ cells using an immunobead method (IMag Cell Separation System; Becton Dickinson, Franklin Lakes, NJ) with 97–99% purity. The EBV DNA load of the fractionated cells was analyzed by real-time quantitative PCR, and compared with that of the PBMCs as a whole.

Clonality analysis of EBV$^+$ cells

The clonality of EBV was determined using Southern blotting with a terminal repeat probe as described previously (Raab-Traub and Flynn, 1986). PBMCs cultured in the presence of 50 units ml^{-1} of recombinant IL-2 were assayed for TCR Vβ expression by flow cytometry (IOTest Beta Mark TCR-Vβ Repertoire Kit; Beckman Coulter, Miami, FL), and examined for EBER using cytosmears.

Labeling for EBER by fluorescence *in situ* hybridization and immunophenotypes

Triple labeling for nuclear EBER and surface phenotypes was performed as described previously (Kimura *et al.*, 2009). Briefly, cells were stained with the appropriate antibodies before fixation and *in situ* hybridization. After fixation with 1% (vol/vol) acetic acid in 4% paraformaldehyde/phosphate-buffered saline for 40 minutes at 4 °C and washing, the cells were resuspended in 45 μl of hybridization solution containing 12 nmol l^{-1} of the FITC-labeled EBER peptide nucleic acid probe, and hybridized for 1 hour at 56 °C. To enhance fluorescence and photostability, the Alexa Fluor 488 Signal Amplification Kit (Molecular Probes, Invitrogen, Carlsbad, CA) was used. Stained cells were analyzed using a FACSCalibur flow cytometer and CellQuest software, version 5.2.1 (Becton Dickinson).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Author contributions

Dr Hirai had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis; study concept and design: Drs Iwatsuki, Yamamoto, Kimura, and Hirai; acquisition and interpretation of clinical and histopathological data: Drs Hirai, Yamamoto, Tsuji, Ito, Kimura, Tsuji, Morizane, Fujii, Suzuki, and Miyake; drafting of the manuscript: Dr Iwatsuki.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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